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CANCER OF SKIN AND INCREASE IN INCIDENCE OF PRIMARY TUMOURS OF LUNG IN MICE EXPOSED TO DUST OBTAINED FROM TARRED ROADS.

J. ARGYLL CAMPBELL.

From the National Institute for Medical Research, Hampstead.

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A PRELIMINARY note of part of this research was published in the *Lancet* of February 3rd, 1934, p. 233. At that time there was definite evidence that dust obtained from tarred roads and containing about 2 p.c. of tar may cause cancer of the skin in mice with metastases, sometimes in the lungs. The experiment with mice is now completed and is recorded herein. Twenty-two guinea-pigs and 4 rabbits, which were also dusted at the same time, are still under observation ; one of these rabbits has developed warts on the right ear. The full data for guinea-pigs and rabbits will not be available for some time.

METHODS.

The mice were exposed to the dust in large respiratory chambers, and the air therein was well mixed by means of a fan, driven by a motor outside the chamber. The carbon dioxide and moisture produced by the mice were absorbed by soda lime spread out in trays at the top of the chamber, while the oxygen used was automatically replaced by almost pure oxygen from a gasometer. These chambers have been described elsewhere (Campbell, 1927*a*, *b* ; 1930).

The mice were separated in groups usually of six or eight, in boxes with perforated zinc lids (area 28 × 21 sq. cm.) ; the holes in the lid were 2 mm. in diameter, approximately half of the lid or 294 sq. cm. being blocked by the unperforated portion of the metal. This had an influence upon the amount of dust reaching the mice in the box.

The dust used was collected as sweepings from tarred roads and then filtered through a sieve with mesh of 100 to the square inch. Most of the dust was obtained thus, but some of it was secured after grinding some of the smaller particles held back by the sieve ; this dust was also filtered as above. A good cloud was produced in the chambers as follows : Some of the dust was placed in a stoppered bottle of 2 litres capacity ; then, with a foot-bellows, a blast of air was directed through a glass tube in the stopper on to the surface

of the dust in the bottle ; in this way a cloud of dust was forced out through a rubber tube leading from the stopper of the bottle into the chamber. The excess air in the chamber was allowed to escape through wash-bottles, connected with outlets situated in the wall of the chamber remote from the entrance for the dust. The finer particles of dust floated in the air in the chamber, and the cloud could be observed for up to about ten minutes. The clouding was observed by means of a 50-watt lamp placed at the back of the interior of the chamber. Such a cloud, as might be produced on a dusty road, was produced about four to six times a day at hourly intervals during five days of each week.

By means of flat dishes placed in the chamber near the mice boxes, the fine dust was sometimes collected as it settled down, and after weighing, it was calculated that 5 to 7 mg. of dust *per diem* reached every square centimetre of the top of the boxes containing the mice. After allowing for the perforations

TABLE I.—*Duration in Days of Dusting and Development of First Wart.*

	Experiment 1 : Dusted mice exposed to CO.	Experiment 2 : Dusted mice.
Period of time over which dusting extended	332	361
Actual number of days of dusting	240	255
Period of dusting before first wart	248	186
Actual number of days of dusting before first wart	180	133

in the lid, it is estimated that at the end of the longest day's dusting there was not more than about 0·9 g. of dust in each box, and that this contained about 20 mg. of tar spread out over the 588 sq. cm., since there was about 2 p.c. of tar in the dust cloud. Each mouse thus probably came into contact with only 1 or 2 mg. of tar every day of dusting. At the end of the day's dusting the mice were transferred to fresh boxes with bedding and food. The clean dust left in the boxes after dusting was removed and used again later. The dust on the hair of the mice was not brushed off, nor was it rubbed in except by the mice themselves.

There were two experiments (see Tables I and III). In one of them (Exp. 2) 73 mice were exposed to the dusting with tar on 255 days during the period from November 17th, 1932, to November 13th, 1933. In the other experiment (Exp. 1) in a similar chamber 85 mice were gradually acclimatized to breathe carbon monoxide up to between 0·20 and 0·30 p.c. ; this acclimatization covered from October 7th to December 12th, 1932. From the latter date till November 13th, 1933, the mice were dusted with tar dust on 240 days as in the other experiment ; but in addition, up to November 29th, 1933, they were shut up in the chamber every evening and continued to breathe carbon monoxide at 0·20–0·30 p.c. all night, and both day and night during every

Saturday and Sunday. They thus breathed carbon monoxide except during the hours of dusting; in this way it was hoped to detect any influence of carbon monoxide, such as might be produced by the carbon monoxide from exhaust gases of motor and factory engines. The mice as a rule were not killed, the great majority being allowed to run their allotted span of life.

For controls there were 65 mice in Group A and 69 mice in Group B. These control mice received the same food (mixture of wheat, barley and oats with a little green-stuff), bedding (sawdust), and water as the experimental animals.

Some details of analyses of tar dust collected from a cloud in the chamber are shown in Table II. Tar was estimated by extracting with benzene at 50° C., evaporating and weighing the residue. Protein was calculated from the total nitrogen, estimate by Kjeldahl's method. The organic matter was

TABLE II.—*Some Details of Analyses of Dust.*

	Experiment 1. (p.c.)	Experiment 2. (p.c.)
Water (120° C.)	1·50	1·26
Organic	7·85	7·01
Tar	1·60	2·80
Protein	1·78 ?	1·78
Rest	3·47	2·43
Inorganic	90·65	91·73
SiO_2	43·33	44·71
CaO	14·92	13·39
Fe_2O_3	3·95	5·40
MgO	3·30	2·15
Al_2O_3	12·34	11·12

estimated from loss due to incineration, while the inorganic substances were estimated by standard methods (Newth, 1907), after fusion with potassium and sodium carbonates, or with calcium carbonate and ammonium chloride. Several analyses of each specimen of dust were carried out, and usually in both experiments the amount of tar was about 2 p.c.; that used for Experiment 2 was usually the higher. As will be seen later, this has a bearing on the results.

Dust from roads contains, besides tar, portions of the road metal and building materials, dust from dried leaves, animal excreta, and oils, etc., from vehicles on the road. There appeared to be little free silica, since there was no reaction in the lung tissue typical of this substance; the silica was thus present mainly as silicates. There was neither lead, zinc nor copper, and only a trace of potassium and sodium.

RESULTS.

Effects on body-weight and growth.—The mice at the start of the experiment were about 2 to 3 months old, and about 17 to 18 g. in weight. The dusted mice in Experiment 2 grew as rapidly as the normal controls. The dusted mice exposed also to carbon monoxide in Experiment 1 did not grow so rapidly during the first 75 days, because they were being acclimatized to the carbon

monoxide. After that time they gained in weight and eventually exceeded the other groups in this respect, until late in the experiment, when both groups of dusted mice lost weight. Fuller details of this aspect of the research and of effects on fertility will be given elsewhere (*Quart. J. Exper. Physiol.*).

Death-rate.—From Tables I and III it will be seen that, by the time the first warts appeared on the skin (186th and 248th day since beginning of dusting), there was a smaller percentage (52) of mice alive in the carbon monoxide group of mice than in the other three groups. This is to be expected as an effect of the carbon monoxide. The control mice of Group B showed a lower survival-rate (58 p.c.) than most normal mice exhibit at this age. This was due to a temporary epidemic of unknown origin in which the liver appeared to be affected; the epidemic disappeared after the 300th day of the experiment. On the 537th day of the research, or when the mice were

TABLE III.—*Survival-Rate: Numbers and Percentages of Mice.*

	Experiment 1: Dusted CO mice.	Experiment 2: Dusted mice.	Controls: A.	Controls: B.
Number of mice at start	85 (100 p.c.)	73 (100 p.c.)	65 (100 p.c.)	69 (100 p.c.)
Number of mice alive after appearance of 1st wart	44 (52 p.c.)	56 (77 p.c.)	50 (77 p.c.)	40 (58 p.c.)
Number of mice alive on 537th day from the beginning of dusting	5 (6 p.c.)	5 (7 p.c.)	18 (28 p.c.)	17 (25 p.c.)
Number of mice alive on 579th day	2 (2 p.c.)	2 (3 p.c.)	10 (15 p.c.)	10 (14 p.c.)

about 600 days old, about 25 p.c. of the control mice were still alive, but only about 6 p.c. of the experimental mice (Table III). This was apparently due to the effects of dusting with the tar dust.

Development of warts and skin cancer.—On the 186th day after commencing the dusting, or after 133 days of actual dusting in Experiment 2, the first wart appeared on the skin of one of the 56 mice surviving at that time (see Tables I and III). In Experiment 1 the first wart did not appear until the 248th day after dusting began, or after 180 days of actual dusting, at which time there were 44 surviving mice. The difference in time of appearance of first warts in the two experiments was probably due in part to the lower percentage of tar used in Experiment 1. It was probably also due in part to the carbon monoxide used in this experiment, since it has been proved (Campbell, 1933) that this gas retards development of warts and skin cancer in mice painted with tar; it also retards rate of growth of transplanted tumours in mice (Campbell, 1932).

Following the appearance of the first warts, subsequent warts developed more or less regularly in both experiments until the end, when there were 25 out of 44 mice, or 57 p.c. (see Table VI), which had typical tar warts in Experiment 1, and 46 out of 56 mice, or 85 p.c., with warts in Experiment 2; the difference is due again in part to the lower percentage of tar in the dust

used in Experiment 1, and also in part to the carbon monoxide breathed by its mice. Table IV indicates that most of the mice had only one or two warts, but a few had four, five or six. Table V shows the site of the warts; it is evident that they were most commonly situated on the head or back. This is to be expected, because the dust on entering the boxes from above would fall first on the head and back. Nevertheless a number of warts were present under the chin; perhaps this was due to one mouse resting its chin on the back of another. Some of the warts remained small, and after a time fell off, leaving a roughened area of skin. Other warts grew rapidly and formed horns an inch long with wide bases. Positions and sizes of warts were charted every week.

TABLE IV.—*Number of Warts on each Mouse.*

Number of warts.	Experiment 1: Number of mice.	Experiment 2: Number of mice.
1	7	19
2	8	9
3	2	9
4	6	3
5	1	4
6	1	2

TABLE V.—*Site of Warts and Cancer of Skin.*

Site.	Experiment 1: Number of mice with—		Experiment 2: Number of mice with—	
	Warts.	Cancer.	Warts.	Cancer.
Head	12	11	24	20
Neck	4	4	4	3
Back	9	8	18	14
Abdomen	1	1	2	0
Limbs	0	0	3	2

As revealed by microscopic examination, typical tar cancer of the skin developed at or near the site of most of the warts. In Experiment 2, 39 out of the 46 mice with warts, and in Experiment 1, 20 out of the 25 mice with warts developed cancer of the skin (see Table VI). This means that, of the 44 mice in Experiment 1 surviving when the first wart appeared, 45 p.c. developed cancer of the skin; and in Experiment 2, of the 56 mice surviving at the time of appearance of the first wart, 70 p.c. developed malignant changes in the skin. It is obvious that mice dying in the experiments before the appearance of the first warts need not be considered in the argument, since cancer requires time for development. None of the control mice revealed cancer of the skin at any time, although one or two of them showed dermatitis (see Table VI).

Tumours of the lungs.—In the lungs of all the dusted mice there was much dust spread out over all the lobes. The tracheo-bronchial lymph-nodes also contained much dust. Although there appeared to be in some cases increase in the lymphocytic elements, there was surprisingly little inflammatory reaction either in the lungs or lymph tissue. The dust did not contain any virulent

infective agent, since there were only two cases of abscess of the lung among the dusted mice, while there was one of the control mice with such a condition. Broncho-pneumonia was present in 8 of the dusted mice and in 1 of the controls, but terminal pneumonic changes were more or less equally distributed.

The outstanding feature was the great increase in incidence of primary lung adenoma in the dusted mice. The histology of this primary adenoma has been described by other workers (Livingood, 1896; Tyzzer, 1907; Murray, 1908; Slye and others, 1914; Murphy and Sturm, 1925). In the present research the tumours varied in size from nodules of 1 mm. in diameter to tumours occupying most of the lobe of a lung. Also they varied from the confined type to the malignant invading type, and there was no means of deciding whether a more or less simple-looking tumour would have remained confined or become malignant later on. There seemed little doubt that the

TABLE VI.—*Details of Incidence of Warts, Cancer and Tumour.*

Experiment.	Total mice.	Mice with skin cancer.	Mice with both tumours of lung and warts on skin.	Mice with tumours of lung only.	Mice with warts only.	Mice with neither tumours nor warts.
1	44	20	21	5	4	14
2	56	39	39	6	7	4
Control A	50	0	0	4	1 (dermatitis)	45
Control B	40	0	1 (dermatitis)	2	2 (dermatitis)	35

simpler tumours gave origin to the malignant type. Fuller details of the histology will be published in a further communication; tumours were obtained similar to those illustrated in figs. 1, 3, 4, 6 and 8 of Tyzzer's (1907) paper, and in figs. 1, 2, 3 and 4 of Murphy and Sturm's (1925) paper. In Experiment 1, 26 (or 59 p.c.) out of the 44 mice surviving after the appearance of the first wart exhibited tumours of the lung (see Table VI); in Experiment 2, 45 (or 80 p.c.) out of 56 mice possessed lung tumours. On the other hand, the control mice showed very much lower incidence of tumours of the lung; 4 out of the 50 mice, or only 8 p.c. of control group B, and only 3 out of 40, or again about 8 p.c. of control group A, exhibited lung tumours. The incidence of the lung tumours in the dusted mice Experiment 2 was tenfold that for the controls. Further, 27 of the 100 dusted mice possessed primary tumours of the lung which were large, and at least half of them of the invading type (see also Slye and others, 1914), whereas only 2 of the 90 control mice possessed similar large tumours. In only 4 of the 100 dusted mice were there metastases in the lung derived from the skin cancer, and primary lung tumours were found in the same lobe with a metastasis.

The primary tumours of the lungs were usually multiple, and more or less equally distributed between the lobes of the right and left lung. Metastases of these tumours in other organs were not observed.

So far as could be estimated from the post-mortem appearances, the warts of the skin and the tumours of the lungs must have originated about the same

time ; certainly the one could not have preceded the other by any great length of time, and no conclusions can be drawn from the relative date of origin.

DISCUSSION.

As regards the cause of the great increase in incidence of the primary lung adenoma, it was easy to find cells containing much dust in the region of the tumours ; but, as there was also much dust in parts of the lungs where there was no tumour reaction, it is difficult to conclude definitely that the primary tumours were due to carcinogenic agents in the dust taken up by the cells. Nevertheless this is the probable cause, because all the dust in the lungs would not contain tar, and all the tar might not contain the carcinogenic agent ; further, only particular cells (*e. g.* cells injured by infection) of the lung may be responsive to the agent, just as only certain cells of an area of skin painted with tar appear to give origin to the malignant growth.

As will be seen from Table VI, most of the dusted mice possessed both cancer of the skin and primary tumours of the lung ; but that the two are not necessarily connected was proved in previous experiments (Campbell, 1933) in which the skin of mice was painted with tar ; although cancer of the skin resulted in about 75 p.c. of these, only about 10 p.c. revealed changes in the lungs, and these were metastases chiefly. Further, some of the mice in the present research possessed tumours of the lung, but no warts or cancer of the skin ; while others showed warts or cancer of the skin, but no tumours of the lung.

Murphy and Sturm (1925) observed primary lung adenoma in up to 78 p.c. of fairly young mice, of which the skin had been painted with pure tar on various different areas, to prevent irritation of the skin and development of skin cancer. Each mouse received 36 applications of tar distributed over 83 days, the area painted being less than 1 cm. Although there were neither warts nor cancer of the skin, the cells of the skin may have been sufficiently irritated to produce carcinogenic agents which may have been carried by the blood-stream to the lungs. A much more probable explanation for the lung tumours, however, is that the mice may have inhaled the carcinogenic agents from dried particles of tar freed from their skin, or from tar droppings mixed with the dust of the bedding in the boxes. Their controls showed a very low incidence (1 to 5 p.c.) of primary lung tumours. Murphy and Sturm's result has a distinct resemblance to that of the present research. They thought that the tar-painting reduced the general resistance to cell growth, and that inhalation of some small foreign particles other than the tar resulted in the irritation of the lung so that tumours developed. They did not think that the tar could become particulate, but the present research indicates that the carcinogenic agent does get into a dust cloud perhaps by being adsorbed on other dust particles.

Some observers employ larger quantities of tar for painting the skin than others, and tars also differ in potency ; therefore dust derived from tar or contaminated with tar must vary in carcinogenic properties. This may explain why some observers (Murphy and Sturm, 1925 ; Schabad, 1928) have obtained a high incidence of primary lung tumours in their painting experiments, while others (Campbell, 1933) have not.

Most previous observers have obtained only about 5 to 10 p.c. of primary lung tumours in normal mice, although Hill (1931) recorded up to about 25 p.c. in some of his small groups on different diets and bedding. Here also the cause of the primary tumours may have been irritation produced by dust from dried food, bedding, etc.

Apart from cancer of the skin and the adenoma of the lung, tumours were scarce in the organs of the dusted mice; only one of them had a mammary carcinoma, and another had a subcutaneous tumour of the foreleg; this tumour had apparently arisen from the epithelium of the skin, from which it had subsequently become separated. One of the controls possessed a mammary carcinoma; tumours were rare in any organ in the controls. Sex did not appear to influence the results described herein.

SUMMARY.

Mice were repeatedly exposed to dust obtained by sweepings from tarred roads and containing about 2 p.c. of tar. Cancer of the skin developed in 70 p.c. of those surviving long enough. The incidence of primary adenoma of the lung was increased to tenfold that of the controls; the lungs of the dusted mice contained much dust. The breathing of carbon monoxide, if anything, retarded the effects of dusting.

The bearing of this research upon the debated increase in tumours of the human lung cannot be assessed at present. The mice were exposed to much more excessive dusting than occurs with human individuals. Cleanliness prevents cancer of the skin in man, and the natural mechanism for removal of dusts from the healthy lung may suffice for the small amounts of dust inhaled.

There is the further question whether these tumours of mice may be compared with those of the human lung. The experiment with mice is to be repeated, and attempts will be made to transplant some of the tumours; the effects of dust, with the tar products removed, will also be studied.

I am indebted to Drs. W. E. Gye and W. Cramer for inspecting sections of some of the tumours; to Dr. H. W. Dudley, F.R.S., for advice on some of the chemical methods; and also to my assistant Mr. C. Pergande for much help in the course of the experiments.

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THE ROUTINE PREPARATION OF SCARLET-FEVER TOXIN OF HIGH VALUE.

R. A. Q. O'MEARA.

From the Wellcome Physiological Research Laboratories, Beckenham, Kent.

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A NUMBER of attempts have been made in recent years to improve the quality of scarlet fever toxin, of which the most noteworthy is that of Hooker *et al.* (1933), who by a special technique obtained toxin with a value of 500,000 skin test doses per c.c.

For some time past, however, the routine toxins prepared in this laboratory have been of considerably greater potency than those described by the authors quoted above, and as the routine preparation of toxin of high value is of considerable practical importance, it is thought that an account of the method which we employ may be of interest.

Theoretical Considerations.

The first essential for good toxin production is a strain of high toxigenicity. The organism which we employ is *Str. scarlatinæ* (Strain Dochez N.Y. 5), which has proved its worth in these and other laboratories.

The second essential is a medium which provides adequate growth and suitable nitrogenous constituents for the elaboration of toxin. Streptococci utilize carbohydrate as their chief source of energy, and are among those organisms which do not cause reversal of reaction. In other words, they rely for their growth mainly upon fermentable carbohydrate, from which they produce relatively large amounts of acid, principally lactic (Langwill, 1924). Unlike such organisms as *C. diphtheriæ*, they do not oxidize the lactic acid which they produce. It therefore accumulates and, as it is a fairly strong acid, causes a rapid fall of pH in an inadequately buffered medium, and so tends to limit growth. It has been found by experience that there is sufficient carbohydrate in horse muscle digest to supply the energy requirements of the organism, so that this type of medium forms a satisfactory basis on which to work. To enrich the nitrogenous constituents of the medium the addition of peptone has been successful, and for the neutralization of the lactic acid formed during growth we employ sodium bicarbonate.

Preparation of the Medium.

The basic medium is a "straight line" 6-hour digest of horse muscle similar to that used by Pope and Smith (1932) for the preparation of diphtheria toxin of high value, but hydrochloric acid is used instead of acetic for acidification prior to boiling. The fermentable carbohydrate is not removed by the addition of yeast when digestion is complete. Instead 1 p.c. of peptone is added, the reaction is adjusted to pH 8·2, and phosphates are precipitated by

adding calcium chloride. Sodium bicarbonate 0·2 p.c. is then added, and the medium is sterilized by filtration through a battery of Berkefeld N candles into 4-litre bottles, which are filled nearly to capacity. A batch of medium prepared in this way may be up to 100 litres in volume, and keeps well in a cool dark place for a period of several weeks.

Toxin Production.

For toxin production a batch of medium which has been prepared in accordance with the above directions is warmed by placing it in the incubator at 37° for 24 hours, and is then inoculated liberally from a vigorous eight-hour culture of streptococci in the same medium. Growth is permitted to continue for 18 hours from the time of inoculation, and the toxin is then harvested by passing first through pulp filters, and later through Berkefeld N candles. The organism gives, in this medium, a constant opacity of six to seven thousand million per c.c., and the reaction falls from an initial pH of 8·2 to an average figure of 6·8.

Constituents of the Toxin.

The chief constituent by which a scarlet fever toxin is judged is the "erythrogenic toxin". It is probable, however, that for the production of scarlet fever sera of greatest therapeutic value, toxins should be employed for immunization in which the other known constituents of hæmolytic streptococcus filtrates, such as the streptolysin investigated by Todd (1932), and the flocculating antigen described by Dyer (1925), receive due consideration. While greatest weight is, therefore, to be given to the erythrogenic toxin, brief consideration of the other components of scarlet fever toxin prepared by the above technique may not be out of place.

Erythrogenic Toxin.

Scarlet fever toxin prepared by our routine method contains on the average a million skin test doses per c.c. In the table are set out the results of an illustrative comparison between our toxins and an American Standard Toxin S.T. 2, containing 35,000 skin test doses per c.c. in parallel with a laboratory standard toxin X 48, containing 30,000 skin test doses per c.c. Two individuals of different degrees of sensitiveness to the toxin were selected, and doses of 0·1 c.c. of S.T. 2, 1/700 dilution (*i. e.* 5 S.T.D.) and 1/3500 dilution (*i. e.* 1 S.T.D.) were injected intradermally. Similar doses of the laboratory standard X 48 1/500 dilution and 1/3000 dilution were injected, and for comparison two toxins, X 641 and X 644, prepared in accordance with the technique outlined in this paper, 0·1 c.c. of 1/100,000 and 1/500,000 dilutions. The reactions were read at 17 hours and 24 hours. The 24-hour readings were in the same order as the 17-hour readings, but by then most of the reactions had begun to fade.

The reactions obtained with 1/100,000 dilutions of X 641 and X 644 were much stronger than any of the others in subject B.

Reference to the table will show that in both individuals the reactions given by 0·1 c.c. of the 1/500,000 dilutions of the routine toxins are of the same order as those given by 1 S.T.D. of the standard toxins. The reactions given by the 1/100,000 dilutions are greater than those given by 1 S.T.D., and

Skin Tests of Various Toxins on Two Individuals "A" and "B".

Dose intradermally 0·1 c.c.

Toxin.	Dilutions.	Skin-test dose.	Reactions (17 hours). A.	B.
$S.T._2$	1/3500	1	15×30 f.	5×7 f.
$S.T._2$	1/700	5	20×30 b.	15×25 f.
X 48	1/3000	1	13×15 f.	10×15 f.
X 48	1/600	5	20×25 b.r.	15×20 f.
X 641	1/500,000	..	17×17 f.	12×15 f.
X 641	1/100,000	..	22×22 b.r.	18×20 b.
X 644	1/500,000	..	18×20	10×15 f.
X 644	1/100,000	..	22×20 b.r.	20×22 b.r.

f. = faint flush. b. = bright flush. r. = raised reaction. Numerals = diameters of reactions in mm.

approach those given by 5 S.T.D. of the standard toxins. The routine toxins therefore contain between one and five million skin test doses per c.c., a potency much in excess of that usually considered satisfactory for a scarlet-fever toxin.

Combining Power.

The combining power of the erythrogenic toxin may be determined in terms of antitoxin by inoculating toxin-antitoxin mixtures intradermally into rabbits. I am indebted to my colleague, Dr. Buttle, for estimating the combining power of these toxins by means of a test, the details of which will shortly be published. He has found that the combining power of the new toxins X 641 and X 644 is 5 to 10 times that of the American Standard Toxin S.T. 2, and of the laboratory standard X 48, prepared by the older methods.

Rabbit-killing Power.

The routine toxins prepared as above have a consistently high lethal value for rabbits when injected intravenously according to the manner of Parish and Okell (1927). A dose of 5 c.c. of these toxins injected intravenously into rabbits over 4 lb. in weight has been found to kill the majority in 48 hours or less.

Hæmolysin.

The usual scarlet fever toxins contain little or no demonstrable hæmolysin of the type described by Todd (1932). In toxins freshly prepared by our routine method this hæmolysin is well represented, and after reduction with sodium hydrosulphite they may be shown to contain, when titrated with sheep cells, up to 50 M.H.D. per c.c., a value little inferior to that obtained by other methods. They have a high combining power value of hæmolysin when titrated with scarlet-fever antitoxin, or antistreptolysin.

Flocculating Substance.

The flocculating antigen produced by streptococci in culture is also represented in these toxins. Their value in this respect is not as high as can be

obtained, when desired, by other methods of culture, but it may be considered adequate for purposes of immunization.

SUMMARY.

A method is described for the routine production of scarlet-fever toxin of very high erythrogenic value. The toxin so prepared contains in addition to an abundance of the erythrogenic factor an adequate amount of the other recognized toxic products of the scarlatinal streptococcus.

Our thanks are due to Dr. McCoy and Surgeon-Commander Veldee for the American Standard Toxin S.T. 2, used for purposes of comparison. We are furthermore indebted to several colleagues in England and America who have very kindly tested our toxin on human beings and animals. Some of the results show that our toxin is of the remarkably high value we have indicated, but in some cases significantly lower values have been returned. These lower titrations are probably due to deterioration caused by the phenol used as preservative in the toxin. This point is under further observation. The deleterious effect of phenol on certain other toxins is well recognised.

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TYPE-SPECIFIC AND GROUP-SPECIFIC SERA AGAINST STREPTOCOCCI.

H. LOEWENTHAL.

From the Hale Clinical Laboratories, London Hospital.

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The problem of producing antibacterial sera of therapeutic value against hæmolytic streptococci is contemplated to-day with some pessimism. Apart from the difficulties encountered in the production of sera with adequate protective properties, it has come to be suspected that since strains differ so greatly in serological respects, only strictly homologous sera could confer protection. Such a situation would make practical serum therapy almost impossible.

Since our expectations of the value of scarlatinal antitoxin in the treatment of septicæmia have not been entirely realized, no effort should be spared to

attack the problem from another angle. Fortunately, some new facts have been discovered during recent years, which enable us to begin again with a more hopeful outlook. These facts are the existence of different colony forms of a single strain of hæmolytic streptococci, which exhibit not only different characteristics of growth, but also different pathogenicity for animals, and above all, different antigenic composition.

Without reviewing the literature, previously done in an earlier paper (Loewenthal, 1932), it should be mentioned here that the different colony forms were discovered by Miss Cowan, that their bearing upon virulence was described by Todd, Lancefield, and Eagles, and finally that Griffith co-ordinated some of his types of scarlatina streptococci with different colony forms.

But the most important fact, that the colony form as well as the character of growth on solid and fluid media (called " Wuchsform " in an earlier paper, 1932) is closely related to the serological components, and that both may change, issued from the works of Andrewes and Christie (1932), and of myself (1932). Whilst Andrewes and Christie made use of the method of gradual absorption, and so succeeded in revealing very small differences in antigenic composition, agglutination and protection tests were used by me.

It was demonstrated that a single strain of a hæmolytic streptococcus may exhibit four different colony forms, the characters of which were described (Loewenthal, 1932). They were termed M, N, O and R. For the present paper N and O only are of interest, M being merely the mucoid variant of N, and R the non-hæmolytic, avirulent, *viridans*-like form. N corresponds to the R of some authors, O to S. These more familiar symbols have now lost much of their original meaning, since they are used for the characterization of virulence rather than of colony surface. Todd's terms " matt " and " glossy " also could not be taken, owing to their bearing upon virulence, for O as well as N can be highly virulent, as is to be described below.

EXPERIMENTAL.

I. *Type-specific Sera (Colony Form N).*

A. *Production of Serum.*

Rabbits were immunized by the intravenous injection of increasing amounts of killed culture for a period of about six weeks. Three injections were made each week of the sediment of 10 p.c. serum broth cultures washed in saline, and resuspended in 0·2 p.c. formalin saline solution. Vaccines killed by heating for half an hour at 60° C. can be used, but streptococci killed by boiling have feeble antigenic properties. The titres of the sera were about 1 : 1240–2480. The whole amount of culture necessary for immunization was about 100 c.c. By a similar procedure sheep were treated, but most of the injections were made subcutaneously, and the course of immunization lasted for more than three months. The amount of culture used was from 1 to 3 litres.

B. *Agglutination.*

With the N form agglutination tests are notoriously difficult, but the obstacle of spontaneous agglutination can be overcome by a method which

follows that of Dochez, Avery and Lancefield (1919). The growth of culture in 10 p.c. serum broth is centrifuged, washed in distilled water, and resuspended in phosphate buffer pH 7·0. The buffer solution is made from N/15 phosphate solutions, and then diluted ten times. The serum itself is diluted with saline, the concentration of NaCl being finally about 0·4 p.c. The object of this procedure is to diminish the concentration of NaCl in a buffered milieu. Agglutination is rapid, and is complete in 2 hours, with the water-bath at 55°. Agglutination also takes place at 37° and is of the granular character. Generally this method worked satisfactorily, yielding indisputable results in nearly 90 p.c. of freshly isolated strains.

The extensive investigation of Andrewes and Christie on agglutination and absorption make it unnecessary to publish in detail my material, which comprises more than 180 strains of hæmolytic streptococci of very different sources. Summarizing, one can say that anti-N sera do not allow a classification of hæmolytic streptococci. Some 5 p.c. of the strains are agglutinated to a low titre (*e. g.* 1 : 40) by non-absorbed anti-N sera, and the remainder are agglutinated nearly to titre (*e. g.* 1 : 1240). But absorption experiments demonstrate that these strains differ widely in their antigenic composition.

Nevertheless anti-N sera are of some value in the identification of strains suspected to be hæmolytic streptococci, because hæmolytic enterococci as well as pneumococci are not agglutinated at all, or only to a very low titre.

c. *Protection.*

Anti-N sera protect mice against infection with 1000–100,000 m.l.d. of the homologous strain. It is necessary to inject the serum on the day before the infecting dose is given. If the serum and the infecting dose are given simultaneously, no protection is detectable. Table I shows one experiment with streptococcus "Krueger". This strain represents a passage strain kept in the Robert Koch Institute, which was first isolated from a case of septicæmia. The serum, prepared by immunizing a rabbit with it, protects against about 100,000 m.l.d. if administered the day before infection.

TABLE I.—*Homologous Rabbit Serum* 771 *Protects against* 100,000 *m.l.d. of* Streptococcus "Krueger", *if given the Day before Infection.*

Amount of culture *Streptococcus "Krueger"* (c.c.).	Control mice receiving 0·5 c.c. normal serum the day before infection.	Mice receiving 0·5 c.c. homologous anti-streptococcus serum—	
		The day before infection.	Simultaneously with infection.
10^{-3}	—	S	D 1
10^{-4}	—	S	D 1
10^{-5}	D 2	S	D 2
10^{-6}	D 2	S	D 2
10^{-7}	D 2	S	D 3
10^{-8}	D 2	—	—
10^{-9}	S	—	—

— = Not tested. S = Survived. D = Died. Numerals indicate the numbers of days before the death of the animal after infection.

There was no protection against other strains derived from cases of septicæmia. Similar results were obtained with a number of other sera, which were produced by streptococci from septicæmia or from purulent infections.

II. *Group-specific Sera (Colony Form O).*

A. *Capsulated Forms.*

(a) *Production of serum.*—The anti-sera are readily produced, and only four or five injections are necessary with quickly increasing doses at intervals of about 4 days. The vaccine can be prepared in the same manner as that for anti-N serum. Yet in this case streptococci killed by boiling also yield vaccines which give rise to protective antibodies as effectively as vaccines killed at 60°, or by formalin. In another respect also this form exhibits a striking similarity to the pneumococci, since it is capsulated, though the capsules are very small compared with those of pneumococci (*cf.* the microphotographs in Etinger-Tulczynska's paper, 1933).

(b) *Agglutination, precipitation and capsular reactions.*—The character of agglutination is somewhat modified by the method employed to obtain the anti-serum. The most typical reaction is obtained by an anti-serum to a vaccine killed by boiling. By this method agglutination in the higher dilutions starts slowly, and is not complete after several hours in the water-bath either at 37° or 55°, but only after standing for some further 20 hours at room temperature. In the lower concentrations the antigen settles down as a coherent disc, like that of the type-specific agglutination of pneumococci. This form of agglutination, as well as the presence of capsules, suggested that the antigen is of carbohydrate nature. Etinger-Tulczynska (1933) followed this suggestion, and succeeded in preparing a substance of carbohydrate character by a very similar method to that used for extracting the capsular antigen from pneumococci. The substance precipitates with the serum of a rabbit immunized against this form of streptococcus, with the formation of shiny droplets and small discs. Capsular reaction, too, can be observed by adding some concentrated serum to a culture : agglutination as well as swelling of capsules follows immediately.

(c) *Protection.*—Sera against capsulated O forms are highly efficient, whether tested by prophylactic or by simultaneous administration. The best titre compares favourably with that of pneumococcal sera. As shown in Table II, 0·2 c.c. of serum protects against 10 million m.l.d. when mixed with the organisms and injected intraperitoneally.

As Table II further demonstrates, the serum does not protect at all against infection by the N form of the same strain. It was possible, however, to produce an effective serum from the N type of this strain which protected against this form, but not against the capsulated form, whether by prophylactic or simultaneous administration. The strain which gave rise to both forms was obtained from a case of septicæmia following mastoid infection.

In all, 8 capsulated O forms were investigated, and protection tests showed them to be identical, since the sera protected as well against heterologous as against homologous strains.

(d) *Experiments on changing colony form and antigenic characters.*—Some

TABLE II.—0·2 *c.c. Rabbit Serum* 942 *Prepared with the Capsulated O Form of Strain* 29 *Protects against 10 million m.l.d. of this form. There is no Protection against the N Form of the same Strain.*

Amount of culture (c.c.).	Mice infected with capsulated O form of strain 29.		Mice infected with N form of strain 29.	
	Mice receiving 0·2 c.c. normal serum.	Mice receiving 0·2 c.c. serum 942.	Mice receiving 0·2 c.c. normal serum.	Mice receiving 0·2 c.c. serum 942.
10^{-1}	—	S	—	—
10^{-2}	—	S	—	—
10^{-3}	—	S	—	—
10^{-4}	—	S	D 2	D 2
10^{-5}	D 1	S	D 2	D 2
10^{-6}	D 2	S	D 2	D 2
10^{-7}	D 2	—	D 3	D 2
10^{-8}	D 3	—	D 3	D 3
10^{-9}	S	—	—	—

— = Not tested. S = Survived. D = Died. Numerals indicate the numbers of days before the death of the animal after infection.

preliminary experiments are here described, which were made with the view of throwing light upon the phenomena of changing one type of streptococcus into another type, both with regard to colony forms and to the intrinsic characters. The presence of capsules as well as of other properties so similar to those of the pneumococci suggested the application to streptococci of the methods of Griffith and other authors for changing one type of pneumococci to another.

Vaccines were made from capsulated streptococci which had been killed by exposure for half an hour to 60°, and were added to broth culture tubes. Antiserum against the strain to be modified was added and also a few drops of blood. This medium was inoculated with one drop of a dilution 10^{-6} of an N strain. In several experiments with different strains we succeeded in changing the inoculated N form into an O form. The time required for the change was generally only a few days. In one case, in which normal serum was contained in the medium instead of immune serum, a fortnight was required. It seems that the change proceeds through the avirulent R form, for the presence of this form could be remarked at about the same time as the first O colonies appeared. Unfortunately these results could not be obtained regularly. In this respect, apparently, the same thing happened when the first experiments with pneumococci were made.

B. *Non-capsulated Forms.*

The capsulated O form only was dealt with in a previous paper, partly because of the unusual characters of this form, and partly owing to the material used, which consisted mainly of highly virulent passage strains in which the O form is particularly likely to occur. Yet the non-capsulated form is the more frequent variant of the non-specific phase of streptococci.

(a) *Production of serum.*—The procedure of serum production against this

form corresponds closely to the method described above for the N form. This method differs from that used for preparing sera against capsulated strains, since it is solely the titre of anti-capsular substance which characterizes the value of the latter sera.

(b) *Agglutination.*—Agglutination also is preferably examined in the same manner as described for the N form, but there is rarely any difficulty with spontaneous agglutination and stability in saline is better. The agglutination proceeds rather slowly in the water-bath at 55°, but is nearly complete after 2 to 3 hours, so that the end-point after further standing at room temperature rises only slightly. In this respect the non-capsulated O form takes a place between the N and the capsulated O form, as well as in regard to saline stability. The agglutination titre of such sera rises to 1 : 2500–5000.

Plain agglutination tests with this form did not reveal many facts on serological relations, but cross-agglutination with absorbed sera proved that a number of types can be differentiated. How many there are cannot be stated yet; but it is quite certain that this O form is definitely more homogeneous than the N.

(c) *Protection test.*—The protective value of these sera attains 100,000 m.l.d. They are protective only when used in prophylactic manner. In this respect they agree with anti-N sera, but there is a striking difference in the important point of specificity. The protective value of sera against the non-capsulated O form is not confined to infection with the homologous strain, but extends to other strains of the same type. As shown in Table III, serum produced with

TABLE III.—0·5 *c.c. Sheep Serum IV Prepared with the Non-capsulated O Form of Streptococcus* 128 *Protects against* 10,000 *m.l.d. of this Strain and* 100,000 *m.l.d. of the Same Form of Streptococcus* 161.

Amount of culture (c.c.).	Mice infected with streptococcus 128.		Mice infected with streptococcus 161.	
	Mice receiving 0·5 c.c. normal serum.	Mice receiving 0·5 c.c. sheep serum IV.	Mice receiving 0·5 c.c. normal serum.	Mice receiving 0·5 c.c. sheep serum IV.
10^{-2}	—	D 2	—	D 1
10^{-3}	D 1	S	D 1	S
10^{-4}	D 2	S	D 1	S
10^{-5}	D 2	S	D 1	S
10^{-6}	D 2	S	D 2	S
10^{-7}	D 3	—	D 2	—
10^{-8}	S	—	D 2	—

— = Not tested. S = Survived. D = Died. Numerals indicate the numbers of days before the death of the animal after infection.

the O form of strain 128 protects against infection with strain 161, as well as with strain 128 itself, the protection titre being 10,000–100,000 m.l.d. The former strain was isolated nearly one year ago from septicæmia following mastoid infection; the latter was recently cultivated from the blood of a case of endocarditis.

It seems almost certain that the very few successful experiments on overlapping immunity recorded by earlier authors (Dochez, Avery and Lancefield, 1919) were performed with the O form.

No protection could be detected by using anti-O serum against N infection.

DISCUSSION.

It has been shown that hæmolytic streptococci of a single strain may exhibit different colony forms. The colonial variants show different degrees of serological specificity. While both may be of high virulence, this dissociation cannot be explained as the degradation from S to R, so well known in other species of bacteria. The appearance of O forms also cannot result from artificial cultivation, for such colonies often appear in the original culture, and it may also happen that the first culture taken from the blood in cases of septicæmia gives rise only to O forms, N forms developing in later subcultures. It is more usual, however, for the N forms to appear first and the O later. There is no doubt, therefore, that both forms represent phases of *Streptococcus hæmolyticus*.

Further investigation is necessary to determine the presence and distribution of both forms in human disease. It seems that changes of form can occur in the course of an infection, and the investigation of the conditions underlying these changes would be of importance. Is it possible to induce this change by immuno-therapeutical or other procedures? The result of such studies might decide the issue of antibacterial serum-therapy, the successful outcome of which now seems to be possible against the O form. Owing to the fact that the production of chronic septicæmic streptococcal infections in animals is very difficult, these problems could be studied better in human disease.

The capsulated O form, against which serum treatment is so promising, was found only occasionally in original culture, but those strains which tended to produce mucoid colonies more often dissociated into the capsulated O form. Milk-borne epidemics of sore throat produced by *Streptococcus epidemicus* ought to be examined from this point of view, for according to those who have investigated these outbreaks the organism tends to produce capsulated forms.

The relation of the capsulated to the non-capsulated O form still requires further examination. It seems almost certain that a single strain can produce both O forms.

The difference in mode of action of sera against capsulated and non-capsulated forms might be explained in this way: the antibodies of sera against capsulated strains attack the bacteria themselves, and render them harmless by neutralization of their capsules; the antibodies of sera against non-capsulated streptococci cannot fulfil any such function, and their mode of action can only be guessed. The serum has to be given in animals some 24 hours before the infecting dose, and this period may be required for its thorough distribution throughout the body, and its effect may be due to some protective action upon the body cells and against the bacterial toxins.

The protection test with mice is not a true index of the therapeutic value

of these sera. Sera which have only a prophylactic effect in mice, which succumb so rapidly, may show a definite curative effect in human septicæmia.

SUMMARY.

1. Strains of hæmolytic streptococci may exhibit different forms of colonies, which differ widely in their serological reactions.

2. The N form is highly specific, as shown by agglutination with absorbed antiserum and by protection tests with mice. The protective value of antiserum therefore is limited to the homologous form.

3. There are two less specific O forms :

(*a*) One is capsulated. Its agglutination, precipitation and capsular swelling reactions correspond to the carbohydrate nature of its capsular substance. Preparation of highly efficient antiserum is possible. All the capsulated forms seem to belong to one serological type.

(*b*) The non-capsulated O form is more frequent. Agglutination as well as protection tests indicate that there are several serological types among this form. Cross-protection can be obtained between organisms of the same type.

4. Both O and N forms can be virulent, and both forms may occur in the primary culture from disease.

5. The prospect of successful serum therapy against the O form seems promising.

The author is deeply indebted to the London Hospital for the hospitality which was extended to him, and to the Academic Assistance Council for a grant which enabled him to resume his work begun at the " Robert Koch " Institute in Berlin. He wishes especially to thank Dr. P. N. Panton for his continued interest and his help in this work.

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THE DETERMINATION OF BLOOD-UREA.

GEOFFREY L. PESKETT.

From the National Institute for Research in Dairying, University of Reading.

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THERE still appears to be lack of a simple, reliable method for determination of blood-urea requiring only small amounts of blood and obviating the use of special and expensive apparatus, yet applicable to a number of samples at a time. The method that is described below has been found useful, and meets these requirements.

The principles involved, namely digestion with urease, distillation of the resulting ammonia with a current of steam in the presence of alcohol and collection in standard acid, have been employed previously by various authors, and all are included in the method which Cole describes in earlier editions of his text-book, but omits from the most recent edition (Cole, 1933). The main disadvantages of Cole's method are : (i) cost of the alcohol required, (ii) necessity of using acid and alkali not weaker than N/20, so that with normal urea content the titration difference is only one quarter of the volume of blood taken, and the error is proportionately large, (iii) difficulty in obtaining correct alkalinity of the digestion mixture before distillation on account of its coloration by blood-pigment. By limiting the volume of the digestion mixture to 2 ml., the amount of alcohol used is reduced to less than one-third and the sensitivity of the end-point in the titration increased, so that N/150 acid and alkali can be employed. If the details of the new method are followed carefully the third difficulty disappears, as the slight warmth of the mixture before distillation flocculates the proteins and leaves a clear supernatant fluid almost free from colour. These and other modifications make it desirable to give a detailed description of the new method, though it closely resembles that of Cole.

Apparatus.

The apparatus used for the distillation is shown in Fig. 1. The distillation (A) and receiving (B) flasks (the former a 500 ml. Kjeldahl combustion flask) and condenser should be of pyrex or resistance glass. The distilled water in the steam flask (C) contains 1 ml. concentrated sulphuric acid ; no further acid is required for further additions of water. Steam delivery is effected by an arrangement of two tubes, one of which slides smoothly into the other. The inner tube (D), which is connected to the steam flask, has an orifice at 0′ in its wall 8–9 cm. from its upper end, the outer tube (E) has an orifice of about the same diameter as that of the tube itself, at 0″ 2·5 cm. above its bend. Tube D is used in three different positions according to the stage of distillation,

namely: (1) drawn out as in Fig. 1, so that alkali can be added to the flask through 0′, (2) pushed in almost to the bend in tube E, for steam distillation, and (3) intermediate, so that steam passes through 0″ but scarcely through the fluid in the flask and cannot escape through 0′, for completion of the distillation after frothing begins. A suitable splash head should be used so that there is no possibility of fluid accumulating in the bulb and passing over into the condenser.

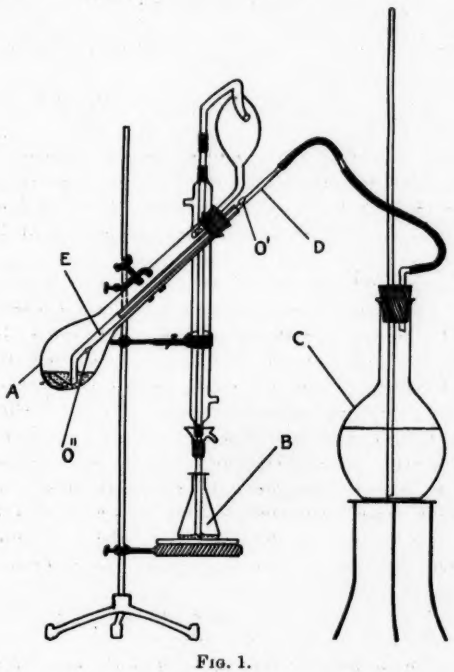

FIG. 1.

Method.

Into a small test-tube (15 × 45 mm.) measure 1 ml. urease solution prepared by grinding one urease tablet (Dunning) in 5 ml. acid potassium phosphate solution (0·3 p.c.). The phosphate is best kept as a 1·5 p.c. stock solution, made with NH_3-free water, and diluted immediately before use. Introduce below the urease solution 1 ml. oxalated whole blood, using a pipette graduated *to contain* 1 ml. Suck up some of the supernatant fluid into the pipette and rinse out with it. Insert a rubber stopper in the tube and mix the contents by a rotatory movement without wetting the stopper. Place in a water-bath at 38° for at least half an hour (a suitable rack in a Wassermann or similar bath is convenient).

While the digestion mixtures are being set up the distillation apparatus should be thoroughly flushed through with a current of steam and then a

supply of freshly distilled, ammonia-free water collected from it for use in rinsing, etc.

When digestion is complete (half an hour suffices for urea values up to 60 mg. per 100 ml. blood), wipe off the water adhering to the outside of the tube and remove the stopper. Transfer the tube to the distillation flask while the latter is warm, so that its contents are not spilled in the neck of the flask. Add 25 ml. of 96 p.c. alcohol containing 1 p.c. of an alcoholic 0·5 p.c. solution of phenolphthalein, and mix. Measure 10 ml. N/150 sulphuric acid and two drops of a saturated alcoholic solution of alizarin (*not* alizarin red) into the receiving flask and place it in position so that the tip of the condenser tube is covered by acid. Connect the distillation flask to the apparatus and, by means of the orifice in tube D, admit just sufficient 4 p.c. NaOH to make the contents of the flask definitely pink. Start the distillation with steam after moving tube D to position 2 ; when frothing commences withdraw the tube to position 3. Four minutes after the first drops of distillate have passed over, lower the receiving flask and, after a further half minute, stop the distillation. The rate of distillation should be such that not more than 50 ml. are collected under these conditions. Rinse the tip of the condenser with a little NH_3-free distilled water and titrate with N/150 NaOH to a faint but definite pink colour. If x ml. N/150 acid are neutralized by the ammonia trapped, then the blood-urea content is $20x$ mg. per 100 ml. This figure on a single determination includes the blank values due to blood and urease solution. For accurate work these should be determined, but for ordinary routine the normal blank values, determined occasionally, can be substituted. The following average blank figures have been obtained : Blood 0·12 ml., urease 0·08 ml. N/150 acid. Values as low as these are only possible if NH_3-free water and 4 p.c. NaOH are used, the latter being boiled thoroughly at the time it is prepared.

In 23 urea determinations carried out recently the difference between duplicates has been 0·036 ml. N/150 acid, in 25 blank determinations 0·026 ml., each expressed as the standard deviation. For normal urea content the accuracy of a single determination is thus approximately ± 2 p.c. In the case of urea (Kahlbaum) added to normal blood 96 p.c. of the theoretical value has been obtained.

Using duty-free alcohol the cost for reagents per 100 determinations is estimated at 7*s*. I have not tested technical methyl alcohol, but should it prove suitable and duty-free alcohol be unobtainable, the cost would then be raised to 12*s*.

Speed and accuracy can be enhanced if microburettes complete with Jena glass reservoirs and soda-lime tubes are available, such as those supplied by P. Haack, of Vienna, in which the acid and alkali keep well. Titration is then conducted rapidly to a point slightly beyond the end-point, and further acid added before completion. With two sets of distillation and receiving flasks titration of one analysis can be conducted during distillation of the next, so as to save further time. An additional convenience is some form of pilot jet on the gas supply to the steam flask, arranged so as to keep the water almost at the boiling-point.

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THE EXPERIMENTAL PRODUCTION OF MUMPS IN MONKEYS.

G. M. FINDLAY AND L. P. CLARKE.

From the Wellcome Bureau of Scientific Research, London.

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NUMEROUS attempts have been made to reproduce mumps in animals since Granata (1908), as a result of experiments on rabbits, first suggested that the ætiological agent was a filterable virus. While cats were employed by Wollstein (1916, 1921), monkeys have more frequently been used as experimental animals. In 1913 Nicolle and Conseil succeeded in producing swelling of the parotid gland in one of three monkeys injected, directly into the gland parenchyma, with material aspirated from the parotids of children suffering from mumps, while Gordon (1914), using a bacteria-free filtrate of saliva from patients with mumps, injected 10 monkeys intracerebrally, and produced in 4 of these animals a meningitis with degenerative changes in the neurones of the cerebral cortex and anterior horn-cells. The condition could not be successfully passaged. One monkey, inoculated intraperitoneally and intravenously, became ill after an incubation period of a week and developed swelling of the parotid glands. More recently conclusive evidence that the cause of mumps is a filterable virus has been brought forward by Johnson and Goodpasture (1934), who, with four out of six specimens of saliva from 6 cases of mumps in the early stages of the disease, obtained in *rhesus* monkeys an acute non-suppurative parotitis analogous to mumps following inoculation of the parotid glands through Stenson's duct.

By chance on the very day that the journal containing the communication of Johnson and Goodpasture arrived in the laboratory, there also arrived tubes of saliva obtained from two children in the early stages of mumps. The saliva had been obtained by washing out the mouth with sterile physiological saline, and had been sent in the ordinary way through the post without any special precautions as to temperature. In the first case (K. V—) the saliva had been collected 72 hours, in the second case (A. G—) 48 hours after the onset of parotid swelling. For these specimens of saliva our thanks are due to Dr. J. L. Dunlop, Deputy Medical Officer of Health for the County of Devon.

EXPERIMENTAL INOCULATION OF MONKEYS.

The saliva from each case was filtered through a British Berkefeld filter, and 4 *rhesus* monkeys were inoculated directly into the left Stensen's duct, each monkey receiving 2 c.c. of filtrate. Monkeys 1 and 2 were inoculated with saliva from K. V—, Monkeys 3 and 4 with saliva from A. G—. Both Berkefeld filtrates were bacteriologically sterile, and on dark field examination failed to show the presence of spirochætes. The technique of injection followed that recommended by Johnston and Goodpasture except that, in order to avoid

excessive salivation, the monkeys, at the suggestion of our colleague, Dr. R. D. Mackenzie, received a subcutaneous injection of atropine sulphate, 1/1000 grain.

Monkeys 1 and 2 did not show any significant rise of temparature during the ensuing fourteen days, and at no time was there any evidence of parotid swelling. They were subsequently reinoculated into the left Stenson's duct, four weeks later, with parotid gland suspension from a monkey with parotitis, and both developed a typical febrile reaction on the 7th and 8th days following injection, accompanied by swelling of the left parotid glands.

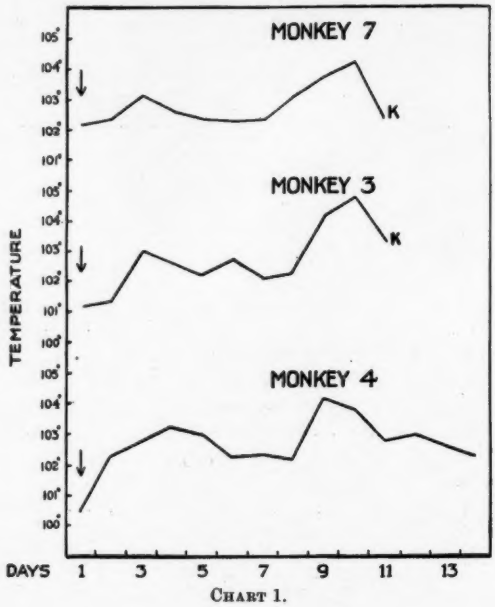


CHART 1.

Monkeys 3 and 4, inoculated with material from A. G—, showed a significant rise of temperature on the 8th and 9th days after inoculation respectively, accompanied by œdema and swelling over the left parotid glands. The temperature charts of these monkeys are shown in Chart 1. Monkey 3 was killed and the parotid gland aseptically removed for further passage, while Monkey 4 was allowed to recover, and was subsequently reinoculated at the same time as Monkeys 1 and 2. It failed to react to this second inoculation, and was therefore presumably immune.

Monkey 3 gland was ground up in physiological saline to form a 20 p.c. suspension, centrifuged at 3000 r.p.m. to throw down gross particles and injected as before into the left Stenson's duct of two *rhesus* monkeys 5 and 6 in 2 c.c. amounts. These monkeys also developed a significant rise of temperature on the 8th and 9th days after inoculation, associated as before with swelling

of the left parotid gland. Altogether six passages have been made in *rhesus* monkeys, involving 9 monkeys, of which 7 have developed a rise of temperature and parotid swelling in from 6 to 10 days after inoculation, the average incubation period for the series being 8 days. The parotid tissues have been bacteriologically sterile, and have failed to show the presence of spirochætes on dark ground examination.

The naked-eye appearance of the swollen parotid glands was very characteristic. The skin over the glands was œdematous and pitted easily on pressure. On reflecting the skin, the parotid gland was seen to be tense and œdematous, the colour reddish purple; sometimes small hæmorrhages could be seen beneath the capsule of the gland. The submaxillary glands appeared normal, and in no case was the parotid of the uninoculated side swollen.

Two monkeys were inoculated into Stenson's ducts with a suspension made from the right parotid gland of Monkey 6, the left gland of which was swollen and œdematous; neither of these monkeys showed any characteristic reaction suggestive of epidemic parotitis.

Two *rhesus* monkeys were also inoculated into Stenson's duct with blood-serum from Monkey 5, each monkey receiving 1 c.c. These monkeys also failed to develop signs of parotitis.

The Leucocyte Count.

In human cases of mumps there is, as a rule, a well-marked leucopenia, involving more especially the polymorphonuclear leucocytes. In monkeys suffering from experimental parotitis Johnson and Goodpasture (1934) have recorded similar findings. The leucocyte changes were investigated in four monkeys during the course of the present experiments, total and differential leucocyte counts being made at intervals of twenty-four hours; the examinations were made in the morning before the monkeys had received their first daily feed. The results were similar in the four animals. The inoculation was followed by a primary leucocytosis, the polymorphonuclear leucocytes being specially increased, then, as seen in Chart 2, there ensued a sudden decrease in the polymorphonuclear leucocytes, followed by a slight increase after the onset of parotid swelling. The small lymphocytes also showed a reduction in numbers, but not to the same degree as the granular leucocytes. The large mononuclear leucocytes did not show any significant variation.

Preservation in Glycerine.

Between the fourth and fifth passages, portions of infected monkey parotid gland remained in 50 p.c. glycerine saline for 5 weeks in the ice-chest at a temperature of approximately 2° C. The virus was still capable of producing parotitis in *rhesus* monkeys when injected into Stenson's duct.

Histological Changes.

The action of the virus of mumps is, as Johnson and Goodpasture (1934) point out, essentially cytotropic. Even when the parotid gland is examined as soon as there is clinical evidence of swelling varying stages of degeneration of the acinar cells can be observed. At first the acinar cells, usually in small scattered groups, become swollen and separated from the basement membrane.

The cytoplasm is granular and the nucleus hyperchromatic. There then occurs an infiltration of mononuclear cells, lymphocytes and plasma-cells; polymorphonuclear leucocytes are conspicuous by their absence. The normal glandular arrangement, already affected by the degeneration of the acinar cells, is still further deranged by the general œdema which involves also the fibrous tissue stroma of the gland, while here and there small hæmorrhages occur.

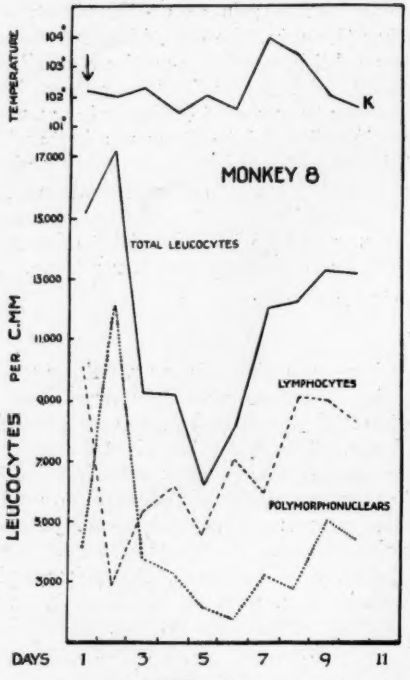


CHART 2.

The infiltrating cells must not be confused with the small masses of lymphoid cells which are sometimes found in the parotids of entirely normal *rhesus* monkeys around the ducts. The cells of the ducts do not show any degenerative changes. No evidence of any cytoplasmic or intranuclear inclusions was

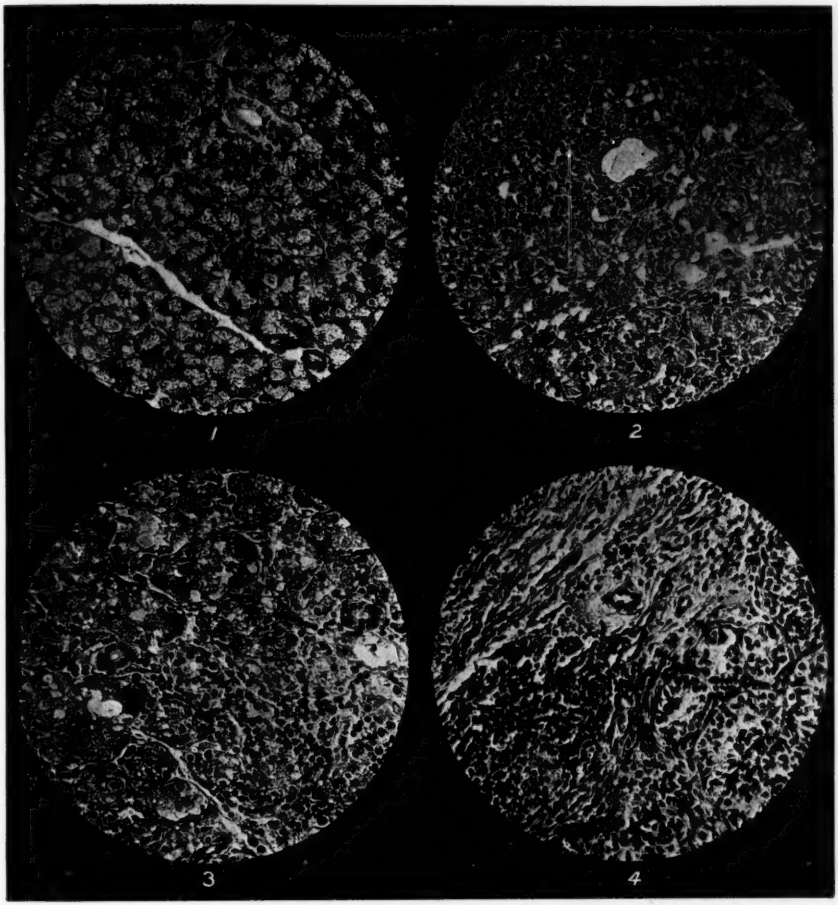
DESCRIPTION OF PLATE.

FIG. 1.—Parotid gland from normal monkey to show the arrangement of the acinar cells and absence of infiltration: stained Mayer's acid hæmalum and eosin. C.B. × 175.

FIG. 2.—Parotid gland of Monkey 6, nine days after inoculation, to show degeneration of acinar cells: stained Mayer's acid hæmalum and eosin. C.B. × 175.

FIG. 3.—Parotid gland of Monkey 8, eleven days after inoculation, to show degeneration of acinar cells and mononuclear infiltration: stained Mayer's acid hæmalum and eosin. C.B. × 175.

FIG. 4.—Testicle of Monkey 9, nine days after inoculation of mumps virus into the tunica vaginalis: infiltration with mononuclear cells. C.B. × 175.



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found either in the acinar or duct-cells. Examination of the submaxillary glands and the parotid on the side opposite to that infected did not reveal any histological changes.

In two *rhesus* monkeys a 20 p.c. suspension of parotid gland after filtration through a British Berkefeld filter was injected in doses of 1 c.c. into the right tunica vaginalis. No appreciable rise of temperature was observed in the monkeys, but on the 8th and 9th days the animals were killed and their testicles examined. In both cases the right testicles were rather congested as compared with the left. Histologically, as seen in Fig. 4, the tunica vaginalis was found to be infiltrated with lymphocytes and plasma-cells which passed in between the more superficial seminiferous tubules. No polymorphonuclear leucocytes were seen. Certain of the seminiferous tubules showed degenerative changes, but the effect on spermatogenesis could not be determined as neither monkey had arrived at puberty.

Intracerebral inoculation of mice, rats and guinea-pigs with a suspension of infected monkey parotid gland failed to give rise to any signs of ill health.

DISCUSSION.

The results obtained by Johnson and Goodpasture (1934) in America are thus confirmed, since it was possible to passage a strain of mumps virus through monkeys for six generations. The fact that saliva from human mumps is infective for monkeys 48, but not 72 hours after the onset of parotitis suggests that human saliva may lose its power of infectivity at a comparatively early stage. The disease produced in *rhesus* monkeys by inoculation of infective material into Stensen's duct is very similar to that occurring in man, while the non-suppurative orchitis caused by the injection of mumps virus into the tunica vaginalis of monkeys, though mild, is an added similarity. The discovery that the *rhesus* monkey is susceptible to mumps should ensure a more thorough study of the virus and the eventual discovery of a successful method of immunization.

CONCLUSIONS.

1. Bacteriologically sterile saliva from a case of mumps 48 hours after the onset of parotitis, injected directly into Stenson's duct, produces in *rhesus* monkeys a mild febrile reaction, swelling of the parotid glands and histological changes very similar to those seen in human mumps. There is also a leucopenia.
2. The virus has been passaged 6 times in monkeys.
3. It can be preserved in 50 p.c. glycerine for at least five weeks.
4. Injection of the virus into the tunica vaginalis of monkeys causes a non-suppurative orchitis.
5. Mice, rats and guinea-pigs inoculated intracerebrally did not develop any symptoms.

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THE ELEMENTARY BODIES OF ZOSTER AND THEIR SEROLOGICAL RELATIONSHIP TO THOSE OF VARICELLA.

C. RUSSELL AMIES.

From the Bacteriological Department, Lister Institute, London.

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ZOSTER has been included for many years among the virus diseases mainly on the ground that no causal organism can be found either in the skin-lesions or in the central nervous system. Additional evidence in favour of this view is afforded by the work of Kundratitz (1925), who claimed to have transmitted the disease to normal children by inoculating them with zoster vesicle fluid known to be bacteriologically sterile. The acidophil intranuclear inclusions found in zoster lesions also suggest a virus origin by analogy with the similar inclusions that form so characteristic a feature of the cytology of some other diseases that are now known to be caused by filterable viruses. Unfortunately all attempts to transmit zoster to animals have failed, so that the usual methods employed to demonstrate the presence of a virus have so far not been applicable. No important advance was therefore made until Paschen (1933) reported the finding of elementary bodies in suitably stained films of zoster vesicle fluid, and noted that these bodies are agglutinated when the vesicle fluid is mixed with zoster convalescent serum. Recently, Taniguchi (1934) has independently reported the finding of elementary bodies in the vesicle fluid of two cases of zoster studied by him.

This observation of Paschen also serves as new evidence of the close relationship of zoster and varicella, since in the latter disease elementary bodies can be demonstrated in large numbers in the vesicle fluid. Furthermore, Paschen has recorded that the bodies become agglutinated when zoster vesicle fluid is mixed with varicella convalescent serum. Clinical and epidemiological evidence of some intimate connection between these two diseases has been steadily accumulating for many years. The more usual sequence is that of varicella occurring among contacts of a case of zoster within the usual incubation period of the former disease, but there appear to be undoubted records of zoster following exposure to varicella. Further evidence is afforded by the experiments of Kundratitz (1925), and Lipschütz and Kundratitz (1925), who obtained a typical chicken-pox eruption in 3 out of 28 children experimentally inoculated with zoster vesicle fluid. Bruusgaard (1932) and Siegl (1927) have repeated these experiments and obtained similar results. Le Feuvre (1917), Low (1919), Kerr (1920), Roxburgh (1927) and Bruusgaard (1932) have collected and reviewed the somewhat scattered literature on the relationship of the two diseases, which at first sight appear to have so little in common.

A few workers have sought for confirmatory evidence of this relationship by serological methods. Netter and Urbain (1924, 1931) claimed that the sera of patients convalescent from either disease would fix complement in the presence of an antigen consisting of a saline extract of the crusts from varicella or zoster lesions. Thomsen (1934), however, has been unable to confirm these findings. Brain (1933) also obtained unsatisfactory results with antigens prepared from crusts, but found that zoster fluid, suitably diluted, gave good fixation in the presence of either zoster or varicella sera, and that the same was true of varicella vesicle fluid. Different samples of vesicle fluid varied in their antigenic potency, and rarely a vesicle fluid was found when tested to be useless as an antigen. Rivers and Eldridge (1929), after demonstrating that chicken-pox vesicle fluid will produce characteristic inclusions when inoculated intra-testicularly into *Cercopithecus* monkeys, used this reaction to determine whether zoster convalescent sera contained antibodies capable of neutralizing varicella virus. They were able to obtain such a neutralizing effect in only one of three sera tested.

Recently the writer (Amies, 1933) has shown that it is possible to obtain relatively pure suspensions of varicella elementary bodies by high-speed centrifugalization of chicken-pox vesicle fluid, and has demonstrated that such suspensions are agglutinated specifically by the sera of patients convalescent from this disease. Paschen's finding of elementary bodies in zoster vesicle fluid suggested that the same technique might be applied to the study of these bodies, and that by means of the agglutination reaction it might be possible to supplement the information already available regarding the relationship of the two diseases.

EXPERIMENTAL DATA.

Clinical Material.

A search extending over six months resulted in the collection of a series of 32 typical cases of zoster. Of these 32 cases, 20 were seen at an early stage of the disease, and, in each of these, stained films prepared from the vesicle fluid revealed the presence of elementary bodies. Any of the methods employed for staining elementary bodies, such as Giemsa or Ziehl Neelsen's carbol fuchsin preceded by Loeffler's mordant, may be used for demonstrating zoster bodies; but the method which gave the best results was the use of Caesares-Gil's mordant and fuchsin in the cold, as recommended for flagella staining by Plimmer and Paine (1921). From a study of films taken at different stages from the same patient it was evident that the bodies occur in large numbers in the fluid removed 24 to 48 hours after the onset of vesiculation. At later stages the number of bodies progressively decreases, and inflammatory cells, mainly mononuclear in type, make their appearance. This cellular invasion is a much later occurrence than in varicella, in which the fluid is usually frankly pustular after 24 hours from the onset. In 3 cases in which films were prepared from fourth-day vesicle fluid the elementary bodies were found to be almost entirely aggregated into large clumps. No attempt was made to obtain an accurate estimate of the size of the bodies, but a careful study of stained

films and of fresh material under dark-ground illumination indicated that they have approximately the same size as the elementary bodies of vaccinia. Elford and Andrewes (1932), by means of the gradocol membrane filtration technique, have estimated the diameter of the vaccinia elementary body to be 0·125–0·175μ.

The series mainly consisted of typical cases of mild "idiopathic" zoster affecting one or two of the cervical or dorsal nerve areas. Three cases deserve special comment. One patient was a child who had been in contact with a case of varicella. This child had a typical band of zoster over the fourth, fifth and sixth thoracic areas, and also a number of small discrete vesicles indistinguishable from chicken-pox lesions scattered over the chest and abdomen, but strictly confined to the same side as that of the zoster lesion. In two other cases the presence of a discrete vesicular eruption was more definite. One of these was a man, aged 57, whose right arm, from the clavicle down to the tips of the fingers, was covered with a confluent bullous eruption. On the right side of the scalp, face and trunk there were many discrete vesicles, containing large numbers of elementary bodies, as did the fluid obtained from the confluent eruption on the arm. In the other case a severe zoster affecting the fourth, fifth and sixth dorsal nerve areas was accompanied by many discrete vesicles on the same side of the scalp, face and neck, but none were present on the nerve areas below. Such cases cannot be correctly designated as concurrent zoster and varicella, since the lesions were in each case strictly unilateral.

A definite history of a previous attack of varicella was obtained in only 2 of these 32 patients; in the remainder no reliable information on this question was available.

Technique of the Agglutination Reaction.

The method of preparing zoster elementary body suspensions was similar to that used in the varicella agglutination reaction (Amies, 1933). Vesicle fluid was removed from the lesions by means of a sterile capillary pipette, and immediately harvested into a small quantity of physiological saline containing 2 p.c. of sodium citrate to prevent clotting. This diluted vesicle fluid was centrifuged at a low speed to throw down any cells which might be present, and then the supernatant fluid was centrifuged at 12,000 r.p.m. for 30 minutes. The supernatant fluid was removed and discarded, and the deposit, which was usually invisible to the naked eye, was resuspended in a small volume of normal saline containing 0·25 p.c. of formalin. This formol-saline was adjusted to a pH of 7·0 immediately before use. After further fractional centrifugation to throw down any clumps of bodies which had not been redispersed, the suspension was, if necessary, diluted with formol-saline until the degree of opalescence approximately matched another elementary body suspension which had been arbitrarily adopted as a standard.

Elementary body suspensions from eight different cases of zoster were prepared, and each of these proved to be sensitive to the homologous agglutinin. The degree of sensitivity varied slightly, depending apparently upon the time at which the vesicle fluid was removed from the lesions. Suspensions prepared from vesicle fluid removed later than 60 hours after the commencement of vesiculation proved unstable, and were therefore useless as antigens for

agglutination. The agglutination test proper was carried out by the hanging-drop technique, the serum and antigen mixtures being maintained at room temperature (average 65° F.), and were examined under the microscope after 24 and 48 hours, using a $\frac{2}{3}$-in. objective and a × 10 ocular. A positive reaction was indicated by the presence of highly refractile irregularly shaped clumps,

TABLE I.—*Agglutination Reactions with Zoster Convalescent Sera and Zoster and Varicella Elementary Body Suspensions.*

Case No.	Number of days from onset of eruption.	Agglutination titre. Zoster suspension.	Agglutination titre. Varicella suspension.
1	20	32	16
2	14	128	64
3	17	128	32
4	18	4	4
5	18	16	16
6	13	64	N
7	19	128	64
8	18	8	N
9	13	128	16
10	14	128	16
11	17	128	16
12	8	N	N
	18	64	32
13	10	—	N
	22	—	N
	32	16	N
14	9	32	8
15	8	8	4
16	21	64	—
17	8	64	N
18	9	128	N
19	17	N	N
20	18	16	N
21	17	8	N
	28	N	N
22	15	4	N
	35	64	16
23	10	N	N
	17	32	16
24	12	N	N
25	10	8	N
26	10	4	4
27	2	N	N
	9	16	16
28	6	N	—
	10	N	—
	16	128	—
	23	>128	16
	31	128	16
29	8	8	—
30	10	16	N
31	21	128	16
32	13	128	16

TABLE II.—*Agglutination Reactions with Varicella Convalescent Sera and Zoster and Varicella Elementary Body Suspensions.*

Case No.	Number of days from onset of eruption.	Agglutination titre. Zoster suspension.	Agglutination titre. Varicella suspension.
1	10	N	32
2	10	16	16
3	14	16	16
4	14	16	16
5	10	8	8
6	7	N	128
	14	N	256
	63	N	N
7	6	N	4
	14	128	128
	56	N	N

N = No agglutination in dilution of 1 in 4, or higher.

which could be shown by appropriate staining methods to consist of aggregates of elementary bodies. Sera obtained from normal healthy persons or from patients suffering from various non-virus diseases were employed as controls. More than 50 such sera were examined during the course of this investigation, and not one of these was found to agglutinate the zoster elementary body suspensions.

RESULTS.

The results of agglutination tests with zoster convalescent sera are summarized in Table I. From these it will be seen that agglutinins could be demonstrated with fair regularity when zoster body suspensions were used as antigen. Thus the sera from 32 different patients gave a positive reaction in all except two cases. One of these failures occurred in the case of a girl, aged 11, who had an extremely mild attack of zoster, while in the other case the patient was a feeble old man, aged 82, whose powers of resistance were probably very low. From the results obtained by testing the serum of the same patients at intervals during convalescence it appears that the process of antibody formation in this disease is a somewhat slow one, so that agglutinins may not be demonstrable until the third week of convalescence. The titres are admittedly not high, but in this connection it should be borne in mind that, as in the precipitin reaction, the relatively large surface area available for adsorption will readily exhaust the serum of its antibody.

The results obtained with zoster sera and varicella body suspensions are less striking than those given by the homologous suspensions. Of 30 zoster sera that gave a positive reaction with the zoster antigen 20 were found also to agglutinate a varicella suspension, the titre usually being slightly lower than that obtained with the zoster suspension. Agglutination tests with varicella convalescent sera gave somewhat variable results. These are summarized in Table II. Of 9 sera that gave good agglutination with varicella body suspensions only 5 gave a positive reaction with a zoster suspension.

DISCUSSION.

The results of these investigations clearly substantiate Paschen's claim that elementary bodies occur in zoster vesicle fluid. It has further been shown that pure suspensions of these bodies are agglutinated specifically by zoster convalescent sera. Unfortunately the lack of a susceptible laboratory animal has made it impossible to employ other methods, such as high-speed centrifugation of virus filtrates or the tissue culture technique, by means of which the ætiological importance of the vaccinia elementary body has been confirmed. It appears justifiable, however, to state that the evidence here presented does at least suggest that the zoster elementary body is the ætiological agent of zoster.

With regard to the relationship of zoster and varicella, it is evident that cross-agglutination tests have not given such uniform results as those obtained with the complement-fixation technique. In spite, however, of the low proportion of successful cross-agglutination tests, it is believed that the high

degree of specificity of the reaction permits one to attach significance to the positive results obtained. In this connection it may be remembered that experiments carried out in this laboratory with suspensions of variola and vaccinia bodies and the corresponding antisera showed an almost complete lack of cross-agglutination (Amies, 1932). Further work on this question has shown, however, that the differences noted are quantitative rather than qualitative, since monkeys hyperimmunized with human variola virus do eventually develop antivaccinial agglutinins and, conversely, hyperimmune anti-vaccinial sera obtained from rabbits will agglutinate variola elementary body suspensions. The failure to obtain cross-agglutination in some of the present series of experiments may perhaps be explained in the same manner.

Recently Craigie (1932) has demonstrated that crude suspensions of vaccinia virus contain a non-particulate Seitz-filterable substance in addition to the vaccinia elementary bodies, and that it is this soluble antigen that is responsible for the vaccinia flocculation (precipitin) reaction. This substance is apparently a product of the elementary bodies themselves, and is probably essentially the same as the heat-stable precipitating substance isolated from autolysates of neuro-vaccinia rabbit testis by Wilson Smith (1932). It seems not unlikely that a similar non-particulate substance may be present in zoster and varicella vesicle fluid, and that complement-fixation reactions with such fluids depend more upon the soluble substance than upon the presence of the intact bodies themselves.

SUMMARY.

1. Elementary bodies morphologically similar to those found in varicella and vaccinia are constantly present in the vesicle fluid of zoster.

2. Pure suspensions of these bodies, prepared by high-speed centrifugalization of zoster vesicle fluid, are specifically agglutinated by zoster convalescent sera.

3. Attempts to demonstrate the relationship of zoster and varicella by means of cross-agglutination tests have met with a fair measure of success.

The majority of the patients examined in this investigation were under treatment at various general hospitals of the Public Health Department, London County Council. For permission to use this clinical material I am greatly indebted to Sir Frederick Menzies, Medical Officer of Health, London County Council, and to the medical superintendents of the various hospitals visited. My thanks are also due to a number of medical colleagues who supplied some of the sera.

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